

**Developing appropriate methodology for assessing anti-pathogen properties  
of mucus-enriched water from corals**

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**Developing appropriate methodology for assessing anti-pathogen properties of  
mucus-enriched water from corals**

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## Abstract

Global coral reef health is in rapid decline. A major contributor to this trend is warming ocean temperatures. As ocean temperature increases, corals become more susceptible to diseases that lead to bleaching and tissue mortality. *Vibrio coralliilyticus* is one of the few documented coral bleaching pathogens. Previous studies developed methods to quantify *V. coralliilyticus* metabolism and developed culturing procedures to test the anti-*Vibrio* potency of mucus-enriched water from numerous coral species. However, the best way to collect and process mucus-enriched water from corals was not determined. Previous efforts obtained mucus-enriched water via coral fragmentation and agitation in seawater. This methodology detected anti-*Vibrio* activity from several species, but required destructive sampling of the corals. This prevents collecting data over multiple time points without confounding time with previous damage. This study evaluates the effectiveness of less destructive methodologies for sampling mucus-enriched coral water. This study evaluated the effectiveness of the less destructive method of slowly sucking coral mucus from colonies in the field using a syringe. Tests using this method on mucus enriched water from 9 species of coral detected no anti-pathogen activity. In contrast, tests using the fragmentation and shaking method found significant anti-pathogen activity in 3 of the 4 species tested. the less destructive method assayed here, is ineffective at assessing the anti-pathogen potential of corals.

## Introduction

Coral reef health has declined severely in recent decades with coral bleaching and mortality occurring more frequently in recent decades, thus minimizing recovery time between stress events (1). Many factors contribute to this deterioration, perhaps most importantly are rising ocean temperatures, ocean-acidification, and overfishing (2). Heightened ocean temperatures result in thermal stress which has been shown to cause coral bleaching and increase coral susceptibility to disease (1,3).

Coral bleaching is the phenomenon where stressed corals lose their microalgal symbionts, zooxanthellae. Corals then often die, exposing the calcium carbonate skeleton (1). *Vibrio coralliilyticus* is a coral pathogen known to cause coral bleaching (3). In addition to being one of the only documented coral pathogens (many other diseases occur, but the causes are not well known), *V. coralliilyticus* is also globally distributed, infects a wide range of coral genera, and virulence factors are expressed when the pathogen is exposed to higher temperatures (4,5). Traits making it an ecologically appropriate study organism. Corals are hypothesized to become more susceptible to *V. coralliilyticus* in hotter water due to thermally-induced microbial dysbiosis (7,8,9). Microbial dysbiosis is a microbiome imbalance where either beneficial microbes are underrepresented or harmful microbes are overrepresented (6,10).

Microbial dysbiosis is observed on coral reefs with an over-abundance of macroalgal cover (6). In a Beatty *et al.*'s 2018 study, two reef states were explored: coral-dominated and algal-dominated reefs. Marine Protected Areas (MPAs) where fishing is limited or prohibited result in abundant herbivorous fishes that keep macroalgal levels low, this is the coral-dominated state (6). Conversely, in fished reefs, grazing pressure on macroalgae is suppressed, allowing macroalgae to bloom and outcompete coral (6). Corals have been hypothesized to manage their

microbiomes via synthesizing antimicrobial peptides, mucus excretion, and symbiosis with microbes that are predatory towards coral pathogens or that produce compounds that defend corals from pathogens (7, 11,12,13).

Mucus-enriched “coral water” is defined as seawater from the reef containing coral mucus (6). The previously utilized methods required fragmentation of the coral sample (6). This resulted in damage to the coral colony itself, potential mixing of internal compounds with surface mucus (which may confound results), and destruction of the sample, resulting in an inability to monitor a coral across multiple time points without confounding effects of the previous physical damage. The intention of this study is to develop a means of sampling coral water without physically damaging the coral, thus avoiding the aforementioned issues.

## **Methods & Materials**

Nine coral species were sampled in Moorea, French Polynesia (*Acropora hyacinthus*, *Acropora pulchra*, *Acropora cytherea*, *Pavona cactus*, *Pocillopora damicornis*, *Pocillopora verrucosa*, *Porites lobata*, *Porites rus*, and *Stylophora pistillata*.) Within each of these nine species, ten separate colonies were haphazardly selected. From each colony, one mL of coral water was collected through a syringe by slowly moving the syringe opening along the coral surface and slowly suctioning water from the coral surface. A corresponding seawater sample was taken about 1m from the coral to use as a control. All corals samples were collected from corals that co-occurred in a 1-3 m deep back reef lagoon on the north side of the island.

Immediately after collection, samples were put on ice in the field and frozen immediately upon return to shore. These remained frozen until they were defrosted and used in the procedures outlined described below.

In lab, 100  $\mu\text{L}$  of coral water treatment or seawater control was aliquoted per well in a 96-well plates, plates were lyophilized using a freeze dryer, and once all liquid had been removed, UV-irradiated for 90 seconds to assure no living bacteria. Each 96-well plate allowed ten samples to be processed at a time, with two technical replicates from each sample. Tetrazolium chloride (TTC) at concentration 0.5  $\mu\text{g}/\mu\text{L}$  and 100  $\mu\text{L}$  of *V. coralliilyticus* suspended in Marine Broth at a concentration of  $10^2$  cells per mL were added to each well. Plates were then incubated at 28°C and metabolism of *V. coralliilyticus* assessed after sixteen and twenty-four hours.

TTC is reduced to triphenyl formazan, a red compound that allows for the measurement of *V. coralliilyticus* metabolic activity by assessing optical density. A BioTek ELx800 absorbance reader was used to quantify this metabolic activity at the sixteen and twenty-four hour time points. Optical densities for blanks containing TTC, Marine Broth, and coral water or seawater, but not containing *V. coralliilyticus*, were taken using the same absorbance reader. These blank absorbances were subtracted from the *V. coralliilyticus* readings to correct for any background absorbance. Coral water metabolisms and seawater metabolisms were compared in a ratio (coral water over seawater) for relative *V. coralliilyticus* metabolism. If the ratio was greater than 1, the coral water tended to stimulate *V. coralliilyticus*; if the ratio was less than 1, the coral water tended to inhibit *V. coralliilyticus*. Statistical evaluation of the coral water and seawater data were compared using the aov and aovp functions of the lmPerm package in RStudio3 to rigorously evaluate which, if any, coral-water collections suppressed or stimulated *V. coralliilyticus* metabolism.

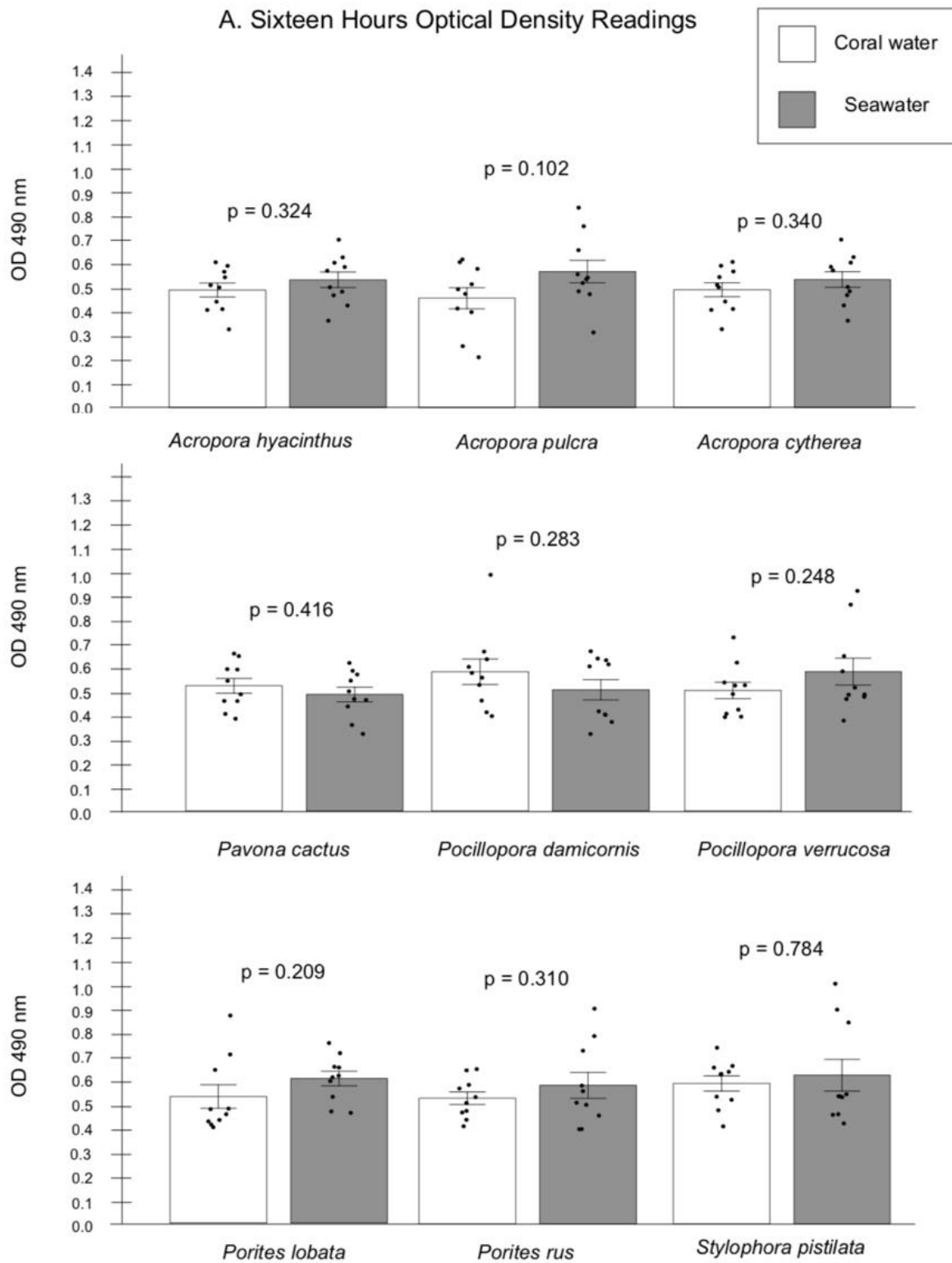
## Results & Discussion

The *V. coralliilyticus* bioassays detected no significant difference between the mucus-enriched coral water treatments and the seawater controls. This result was consistent across all nine coral species, and after both 16 and 24 hours of growth (Figure 1.)

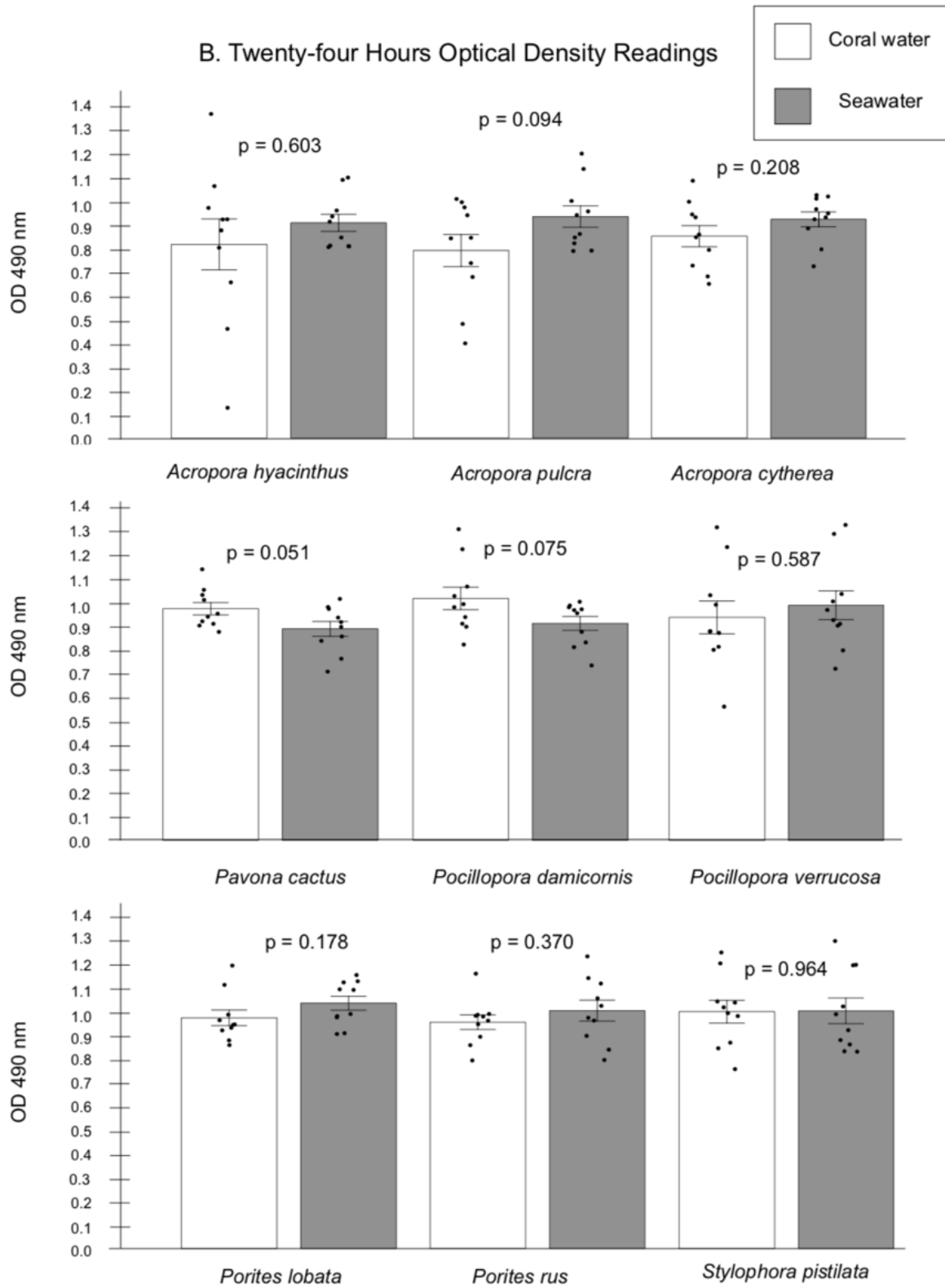
Comparatively, a 2017 project (Figure 2) used the fragmentation method to obtain coral water from four species in Moorea, French Polynesia. These results showed that three of the four coral species (*Acropora pulchra*, *Pocillopora verrucosa*, and *Porites rus* - all of which were included in our later assays of nine species noted above) significantly inhibited *V. coralliilyticus* growth at both 16 and 24 hours, and *Pocillopora damicornis* was significantly stimulatory towards the pathogen at 24 hours. If the new methods explored in this study had worked, we would have expected to see this pattern recreated in the samples of these species.

These findings may have resulted from the syringe methods not capturing enough mucus relative to seawater to produce the results that matched the fragmentation methods results. Alternatively, the portions of the coral, its microbiome, or its symbiotic zooxanthellae that produce the anti-*Vibrio* effects may be associated with internal coral tissues and not present on the coral surface. Modifying the methodology to increase the amount of mucus sampled may yield better results.

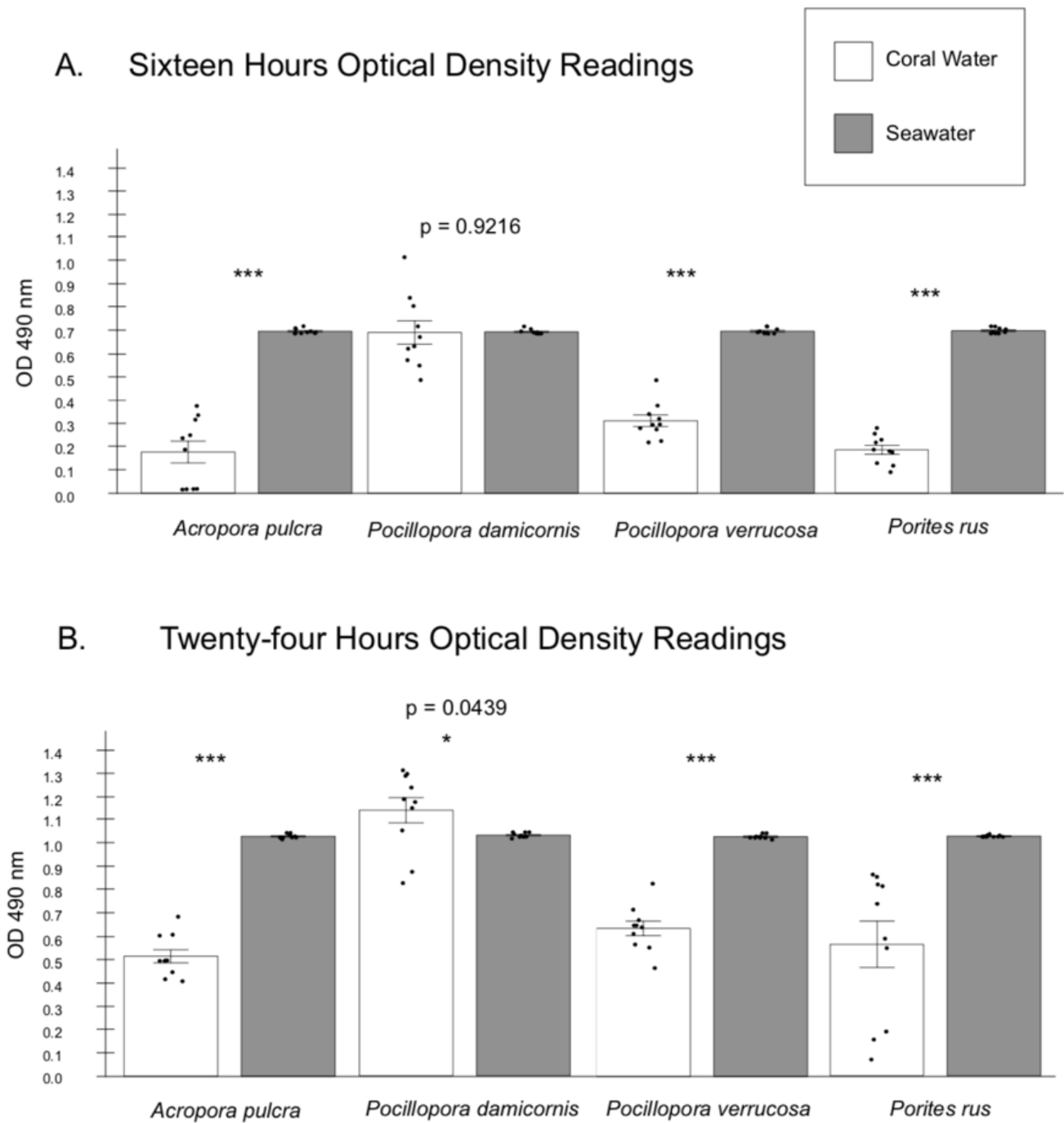




**Figure 1 (A). Coral water treatments and seawater control optical densities compared at 16 hours.**



**Figure 1. (B) Coral water treatments and seawater control optical densities compared at 24 hours.**



**Figure 2. Coral water treatment and seawater control optical densities, using the fragmentation method, compared at 16 and 24 hours.**

Future work involving lightly agitating the corals prior to collecting the coral water should be conducted. Methods to be tested next include mild abrasion of the coral surface (brushing the coral surface with a toothbrush which might mimic abrasion from moving seaweeds or sand scour) before using the syringe method to collect the coral water or exposing corals to air and collecting the mucus that is subsequently produced in response to UV and air. Developing a method that allows for data collection across multiple time points, and treatments, is crucial to understanding the dynamics of different corals species' ability to inhibit *V. coralliilyticus*, and how this ability may be induced or compromised by the various biotic and abiotic stresses corals experience on reefs in the Anthropocene.

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